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14. ABSTRACT

XIAP is a promising new molecular target for the design of an entirely new class of cancer therapy to improve survival and quality of life of prostate cancer patients. New therapies targeting XIAP may prove to be especially effective to overcome apoptosis-resistance of prostate cancer cells. Using a powerful computational structure-based database screening, we have discovered the only class of non-peptide small-molecule inhibitor of XIAP. Through computational design and chemical modifications, we have now obtained very potent small-molecule inhibitors of XIAP. They bind to XIAP with nanomolar affinities and effectively inhibit cancer cell growth in PC-3 human prostate cancer cell line. These compounds represent promising leads for further optimization toward our ultimate goal of developing a new class of anticancer drugs by targeting XIAP and promoting apoptosis in cancer cells.

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Introduction

Androgen withdraw remains the only effective form of systematic therapy for men with advanced prostate cancer, with objective response in 80% of patients. Unfortunately, progression to androgen independence occurs within a few years in the majority of these cases. Despite extensive clinical trials, chemotherapy has limited antitumor activity, with an objective response rate of less than 50% and no demonstrated survival benefit. Thus, androgen-independent (hormone-refractory) disease is the main obstacle to improving the survival and quality of life in patients with advanced prostate cancer. There is an urgent need for novel therapeutic strategies for advanced prostate cancer by targeting the fundamental molecular basis of resistance of androgen-independent disease to chemotherapy.

Most of the current chemotherapeutic agents for advanced prostate cancer work by indirectly inducing programmed cell death or apoptosis in cancer cells. The aggressive cancer-cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways. Such alterations include an impaired ability of the cancer cell to undergo apoptosis. Indeed, hormone-refractory prostate cancer is very resistant to apoptosis induced by chemotherapeutic agents and radiation. Thus, current and future efforts toward designing and developing new therapies to improve survival and quality of life of prostate cancer patients must include strategies that specifically target prostate cancer-cell resistance to apoptosis. Therefore, developing new and specific anticancer drugs that target critical apoptosis regulators by overcoming apoptosis of prostate cancer cells is a very exciting and fruitful area of research to improve the outcome of current anticancer therapies.

Inhibitor of apoptosis proteins (IAPs) have been identified as a class of central negative apoptosis regulators. XIAP is the most potent anti-apoptotic member among all the IAPs and has a key function in the negative regulation of apoptosis in both the cell surface death receptor- and the mitochondria-mediated pathways. Prostate cancer PC-3, DU-145 and LnCap cell lines have much higher levels of XIAP protein than normal prostate epithelial cells. XIAP has been implicated to play a key role in apoptosis-resistance of prostate cancer cells to chemotherapies. Because XIAP blocks apoptosis

at the effector phase, a point where multiple signaling pathways converge, new therapies targeting XIAP may prove to be especially effective to overcome apoptosis-resistance of prostate cancer cells and to develop an entirely new class of cancer therapy to improve survival and quality of life of prostate cancer patients.

Smac/DIABLO is a potent pro-apoptotic protein, which directly interacts with XIAP and other IAPs and promotes apoptosis by antagonizing the anti-apoptotic function of IAP proteins. Micro-injection of Smac protein was shown to promote apoptosis in prostate cancer cells. Three recent studies showed that short Smac-based peptides, consisting of the first 4 to 8 residues of the N-terminus of Smac tethered to a carrier peptide for intracellular delivery, sensitize various tumor cells *in vitro* for apoptosis induced by TRAIL or chemotherapeutic drugs and greatly enhance the anti-tumor activity of therapeutic agents *in vivo*. Importantly, Smac-based peptides show little or no toxicity to animals. These studies thus provide the important proof-of-concept that Smac-based small molecule inhibitors may have a great therapeutic potential for treating prostate cancer with XIAP overexpression.

Peptide-based inhibitors have several intrinsic limitations to be developed as potential drugs, including poor cell-permeability and poor *in vivo* stability. For this reason, in this IDEA Development Grant, we propose to design and synthesize potent, non-peptide**,** cell-permeable, small molecule inhibitors of XIAP (Smac mimetics) and to test their therapeutic potential for the treatment of prostate cancer using a powerful structure-based design strategy based upon a class of most promising non-peptide small molecule inhibitors we have already discovered in our laboratory. Successful carried out, our studies will represent an exciting step and lay the foundation for developing an entirely new class of anticancer drugs by targeting a central apoptosis regulator protein. It is predicted that such a drug will have very few side effects and will be able to significantly improve the outcome of current clinical treatment protocols by specifically overcoming apoptosis-resistance of prostate cancer cells to chemotherapeutic agents through targeting the fundamental molecular basis of apoptosis-resistance in prostate caner cells.

Body of the report:

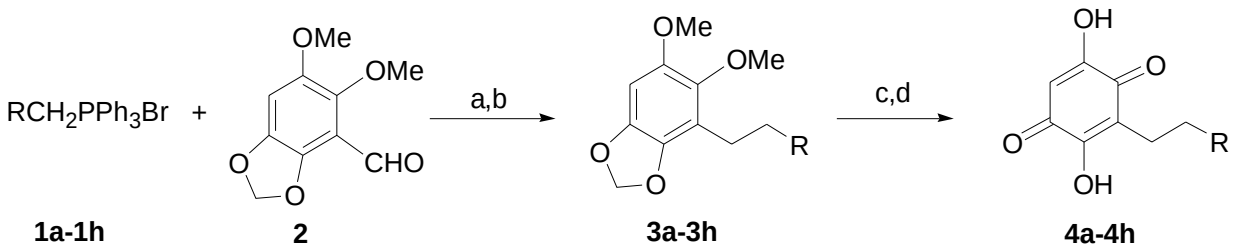
Through structure-based database searching, we have previously discovered embelin as a non-peptide, small-molecule inhibitor of XIAP. Embelin was determined to bind to the XIAP BIR3 domain with an IC_{50} value of 4.7 μM (K_i = 400 nM, Table 1) in our optimized, competitive fluorescence-polarization (FP)-based assay. To the best of our knowledge, embelin is the only known class of non-peptide inhibitor that binds to the XIAP BIR3 domain, whose chemical structure is not related to the AVPI peptide in Smac. Hence, embelin represents a promising initial lead for optimization toward our ultimate goal of developing a new class of anticancer drugs to target XIAP. We have therefore pursued design, synthesis and evaluation of new embelin analogues to improve their binding affinities to XIAP and other activity in inhibition of cell growth and induction of cell death in human prostate cancer cells with high levels of XIAP.

Embelin consists of the dihydroxyquinone core and a long hydrophobic tail. Our modeling suggested that the hydrophilic dihydroxyquinone core forms a number of hydrogen bonds with XIAP and the hydrophobic tail interacts with hydrophobic pocket where the isoleucine residue in the AVPI Smac peptide binds. Our initial modifications focused on the hydrophobic tail portion of the molecule.

A series of embelin analogues **4a**-**4h** with different hydrophobic tails were designed and synthesized. The synthesis of compounds **4a-4h** is shown in **Scheme 1**. Briefly, commercially available, different phosphonium salts **1a-1h** were treated with 1:1 equivalent of *n*-butyllithium, followed by reaction with aldehyde **2,** which was prepared according to a published method. Hydrogenation afforded the key intermediates **3a-3h**. The final target compounds **4a-4h** were obtained by the oxidation of **3a-3h** with ceric

ammonium nitrate, followed by hydrolysis with 70% perchloric acid and concentrated hydrochloric acid.

Scheme 1. Synthesis of designed new embelin analogues.



1a-1h **2** **3a-3h** **4a-4h**

Reagents and conditions: (a) n-BuLi, THF, 0 °C, 10 min; (b) H2, 10 % Pd-C, EtOAc, room temperature; (c) CAN, CH3CN-H2O, 0 °C, 1 h; (d) HClO4, HCl, dioxane, room temperature, 48 h.

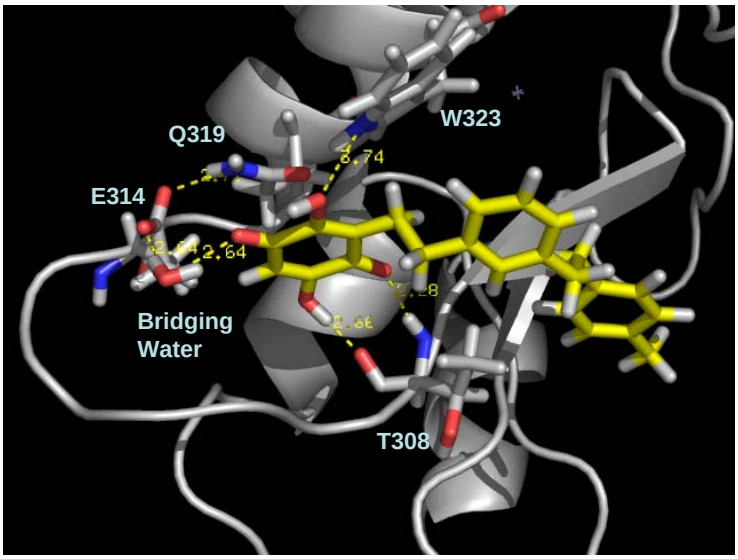
Compounds **4a-4h** were tested for their binding affinities to recombinant XIAP BIR3 protein using our established quantitative fluorescence-polarization(FP)-based competitive binding assay and compared directly to embelin (**1**) and the Smac AVPI peptide. The results are summarized in Table 1.

Compound **4a** was designed to test the importance of the $C_{11}H_{23}$ long hydrophobic tail, in which the $C_{11}H_{23}$ tail was replaced by an ethyl group. Our FP-based binding assay showed that compound **4a** has a K_i value of 10.4 μM to XIAP BIR3, thus 25-times less potent than embelin. This suggests that the $C_{11}H_{23}$ long hydrophobic tail is critical for the binding of embelin to XIAP BIR3. Based upon this result, we have designed and synthesized compound **4b** with a *n*-octyl side chain. Compound **4b** has a K_i value of 1.25 μM. Thus, although compound **4b** is 3-times less potent than embelin, it is 8-times more potent than compound **4a,** further confirming the importance of the long hydrophobic tail in the binding of embelin to XIAP BIR3.

The crystal structure of XIAP BIR3 in complex with Smac showed that the binding of Smac to XIAP BIR3 is mediated by AVPI four amino acid residues in Smac and a well-defined surface binding groove in XIAP BIR3. While the alanine residue in the Smac AVPI binding motif forms an extensive hydrogen bonding network with XIAP BIR3, the proline residue has hydrophobic contacts with Trp323 in XIAP. Our modeling suggested that the hydrophilic dihydroxyquinone core in embelin mimics the alanine residue in the Smac AVPI peptide to form a network of hydrogen bonding network. We have designed two new analogues, **4c** and **4d**, to explore whether a phenyl ring would be able to mimic the interaction between the proline ring in the Smac AVPI peptide and Trp323 in XIAP. As can be seen, while compound **4c** with a $(CH_2)_4$ linker between the dihydroxyquinone group and the phenyl ring has a K_i value of 1.3 μM, compound **4d** with a $(CH_2)_2$ linker has a K_i value of 0.71 μM. We have therefore made additional modifications based upon compound **4d**.

In the Smac AVPI peptide, the isoleucine residue binds to a hydrophobic pocket and plays an important role for the binding of the AVPI peptide to XIAP BIR3. Our modeling suggested that the long hydrophobic tail in embelin interacts with this hydrophobic pocket in XIAP. This is further supported by the binding data of embelin and compound **4b**. We have thus designed and synthesized a series of new analogues based upon compound **4d** in an attempt to capture the hydrophobic interaction between the isoleucine residue in the Smac AVPI peptide and the hydrophobic pocket in XIAP BIR3.

Figure 1. Model between XIAP-BIR3 and **4h**



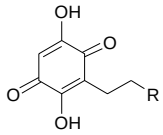
Compound **4e** with a n-butyl group attached to the *meta*-position on the phenyl ring in compound **4d** has a Ki value of 0.48 μM, which is more potent than **4d**. Encouraged by this result, we replaced the butyl group with an ethylphenyl group since it was previously shown that replacement of the isoleucine in the Smac AVPI peptide by a phenylalanine residue increased the binding affinity of the resulting peptide. This resulted in compound **4f**, which has a K_i value of 0.38 μM binding to XIAP. Thus, compound **4f** is as potent as embelin.

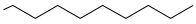
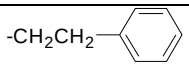
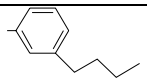
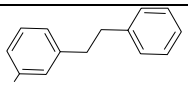
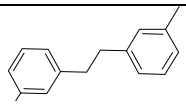
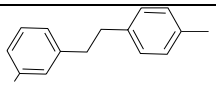
Two additional compounds were designed and synthesized to further explore the interaction between the terminal phenyl ring and XIAP by installation a methyl group either on the *meta*- or *para*-position. The resulting compounds **4g** and **4h** have Ki values of 180 nM and 140 nM, respectively. Hence, compound **4g** and **4h** are more potent inhibitors of XIAP than embelin. The binding model predicted for compound **4h** in complex with XIAP BIR3 domain is shown in Figure 1.

We have tested these compounds for their activity to inhibit cancer cell growth in human PC-3 human prostate cancers using 4-day standard WST-based assay. The results are summarized in Table 1. As can be seen, consistent with its high binding affinity, compound **4g** potently inhibits cell growth in PC-3 cancer cells with an IC50 value of 9.1 μM.

In summary, embelin represents a novel class of non-peptide small-molecule inhibitors of XIAP. Through computational design and chemical modifications, we have now obtained very potent small-molecule inhibitors of XIAP. For example, compounds **4g** and **4h** have K_i values of 180 nM and 140 nM, respectively for binding to XIAP. In addition, compound **4g** is effective in inhibition of cancer cell growth in PC-3 human prostate cancer cell line with an IC_{50} value of 9.1 μM. Hence, compound **4g** represents a promising lead compound for further optimization toward our ultimate goal of developing a new class of anticancer drugs by targeting XIAP and promoting apoptosis in cancer cells.

Table 1. Binding affinities to the XIAP BIR3 in an FP-based binding assay for **4a-h**



Compounds	R	K_i ± SD (μM) FP-Based Binding Assay	IC_{50} (Inhibition of cell growth in PC-3 cells)
1		0.40 ± 0.13	8. 0
4a	H	10.4 ± 1.3	128
4b	$-CH_2CH_2CH_2CH_2CH_2Me$	1.25 ± 0.9	52. 0
4c		1.3 ± 0.3	16. 4
4d		0.71 ± 0.17	21. 8
4e		0.48 ± 0.3	15. 8
4f		0.38 ± 0.09	14. 3
4g		0.18 ± 0.09	9. 1
4h		0.14 ± 0.05	16. 3

Key Research Accomplishments:

(1). We have designed and synthesized a novel class of non-peptide small-molecule inhibitors of XIAP. The two most potent lead compounds, **4g** and **4h,** bind to XIAP BIR3 with K_i values of 180 and 140 nM, respectively.

(2). Compound **4g** potently inhibits cell growth in PC-3 androgen-independent human prostate cancer cell line**.** Hence, compound **4g** represents a promising new lead compound for further optimization.

(3). Extensive optimizations are being carried out based upon compound **4g**.

(4. Extensive *in vitro* and *in vivo* studies are being carried out for compound **4g**.

Reportable Outcomes:

(1). A manuscript described the design, synthesis and biochemical and biological characterization of this class of compounds has been finished and will be submitted shortly.

Conclusions: Embelin represents the only known class of non-peptide small-molecule inhibitors of XIAP discovered to date. Our design and optimization have yielded new analogues of Embelin with high binding affinities to XIAP. In addition, the most potent new analogue is effective in inhibition of cell growth in human prostate cancer cells with high levels of XIAP. Further optimization for this class of compounds and extensive testing may ultimately lead to an entirely new class of anticancer therapy for the treatment of androgen-independent human prostate cancer by targeting XIAP.